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Review

The polymerase chain reaction: a new tool for the understanding and diagnosis of HIV-1 infection at the molecular level

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The polymerase chain reaction (PCR) is at present the most powerful analytical tool for detection of specific nucleic acid sequences. The method is based on the *in vitro* amplification of DNA segments before detection with conventional hybridization techniques or visualization following electrophoresis and staining. The current diagnostic methods for HIV-1 do not allow easy identification of subgroups of infected patients including infants born to seropositive mothers, individuals with delayed serological responses to the virus, infected patients with indeterminate serology results, and patients with dual retroviral infections. Furthermore, response to antiviral therapy cannot be evaluated with serological assays. The rationale for applying PCR in those situations is elaborated here. The applications of this technique for HIV-1 as a diagnostic test and for the understanding of the pathogenesis of this retrovirus are described. Potential limitations of this technique for diagnostic purposes include mainly the possibility of false-positive results due to contamination and false-negative reactions caused by *Taq* polymerase inhibition. Non-isotopic means for detection of amplified products have been described and should allow for a wider application of this technology. Modifications of PCR which make use of internal standards seem promising for quantitative analysis of nucleic acids. PCR has great potential for viral diagnosis but still requires further studies and better characterization.

KEYWORDS: HIV-1, PCR, hybridization, viral diagnosis, AIDS, non-isotopic probes

INTRODUCTION

A human retrovirus, the human immunodeficiency virus type 1 (HIV-1), is the etiological agent of the acquired immunodeficiency syndrome (AIDS).[1,2] The spread of this virus has caused a world-wide epidemic. The median survival time of patients after the first symptoms of AIDS is 12 months, with a probability of survival 3 years after the first manifestations of AIDS of 22%.[3] Major efforts have been invested in the development of efficient diagnostic tools for the prevention, control and management of this deadly disease. The elusive nature at first of the causative agent of AIDS and the difficult propagation procedures required for isolation of retroviruses have complicated the search for such assays.

Since its discovery in 1983, HIV-1 has been cloned and sequenced.[1,4–6] The complexity of the 9·2 kb RNA

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genome is characteristic for lentiviruses and encodes[7] not only for reverse transcriptase, viral envelope and capsid proteins, but also for numerous regulatory proteins.[7] The genomic organization as well as the replication cycle of the virus have been described elsewhere.[7-9] The pathogenesis of HIV-1 infection will be briefly outlined here.

HIV-1 preferentially infects cells with the CD4 receptor including, T-helper lymphocytes, monocytes, macrophages and glial cells.[10] In the infected cell, the outer proteins of the virions are removed to liberate the viral RNA and RNA-dependant DNA polymerase (reverse transcriptase). A linear unintegrated cDNA copy is synthesized with the viral reverse transcriptase from the HIV-1 RNA template by a complex process.[7,8] The double-stranded HIV-1 cDNA copy can circularize, translocate into the nucleus and randomly integrate in the host chromosome. This proviral DNA then serves as a template for the synthesis of specific mRNA or viral RNA for new virons.[8] Unintegrated provirus can also accumulate and cause cytopathicity[11] or may result in a latent infection.[12] Resting lymphocytes are not permissive for replication of HIV-1.[13] However, immunological activation of host CD4+ cells or possibly, coinfection with another activating virus, result in induction of HIV-1 replication.[14] This process leads to a productive infection and cell death. The destruction of key cells of the immune system increases susceptibility to opportunistic pathogens and malignancies.[10]

CONVENTIONAL DIAGNOSTIC METHODS

The laboratory diagnosis of HIV-1 infection has relied on serology, viral antigen detection and viral culture.[15] Serology identifies infected patients by measuring the presence of the host immunological response to HIV-1 proteins. Repeatedly positive samples require confirmation with immunofluorescence, radioimmunoprecipitation or Western blot. The serological tests are the cornerstone of routine blood screening and have been adapted to convenient and widely used assay formats such as enzyme immunoassays (EIA) and latex agglutination tests.[15,16] However, serological assays provide limited information in selected clinical situations.

Serology does not correlate with progression of disease except for the decline in anti-core antibodies with AIDS.[17] Serology assays are difficult to interpret in infants born to seropositive mothers because of the lack of a reliable HIV-specific IgM test and because of passive transplacental passage of maternal IgG antibodies to the infant in the absence of true neonatal infection. A blunted antibody response has also been described in some infants.[18] Antibodies against HIV-1 can also be passively acquired by transmission through unscreened blood transfusions, although the risk of acquiring the virus by this route is also important. Some studies have demonstrated a delayed seroconversion in some individuals who may harbour the virus for periods sometimes exceeding 6 months.[19-21] Transmission of blood through transfusion donors who had not yet seroconverted to HIV-1 has been reported.[22] Response to antiviral therapy cannot be evaluated by serology. Thus, a sensitive direct detection system for HIV-1 in biological fluids would be desirable, especially in the investigation of the clinical situations enumerated above.

Isolation of HIV-1 by co-cultivation of the patient's peripheral blood mononuclear cells (PBMC) with stimulated PBMCs from uninfected donors can be accomplished but requires specialized laboratories, is labour-intensive, can be negative in seropositive asymptomatic patients and requires prolonged periods of time before the development of positivity.[23-25] The p24 antigen of HIV-1 can be detected by a commercial EIA in serum, plasma and cerebrospinal fluid (CSF).[15,26] This assay was shown to be relatively insensitive in latent infection without active replication of HIV-1.[15] A new category of diagnostic assays, nucleic acid hybridization techniques, provide an alternative for the diagnosis of infectious diseases.[27] However, assays relying on the detection in samples of HIV-1 proviral DNA or viral RNA by hybridization were shown to be insensitive.[11,28] Cytoplasmic dot and *in situ* hybridization could identify positive lymphocyte co-culture samples more efficiently than reverse transcriptase and antigen detection assays but still required viral culture.[29,30] Although HIV-1 RNA was demonstrated in 80% of seropositive subjects in one study.[11] Shaw *et al.* could detect HIV-1 DNA sequences in PBMCs of only one out of 15 AIDS patients with Southern blot hybridization,[11] while Harper *et al.* detected viral RNA with *in situ* hybridization in PBMCs of seven out of 14 AIDS/AIDS-related complex (ARC) patients.[28] These techniques also identified HIV-1 in brain macrophages[32] and lymph nodes.[28,33]

Thus conventional hybridization assays needed to reach a better sensitivity level to be helpful in HIV diagnosis. In this review, we will discuss the potential applications of a new methodology for gene analysis, the polymerase chain reaction (PCR), as a direct method for detection of HIV-1 nucleic acids. The amplification of specific DNA sequences with PCR increases the amount of nucleic acids to levels detectable by standard hybridization assays. The study of latently infected patients with a low viral copy number is thus made feasible.

POLYMERASE CHAIN REACTION

Principles of PCR

To solve problems caused by the relatively poor sensitivity of direct nucleic acid detection tests, a different approach was evaluated in which nucleic acids present in clinical samples were specifically amplified before detection with hybridization assays. PCR is an *in vitro* amplification method of DNA[34,35] resulting in selective enrichment of specific sequences up to a million fold.[36] This method can detect one gene copy in 10^6 genomes.[36] Small amounts of DNA can be amplified by means of repeated cycles of DNA synthesis with a DNA polymerase and oligonucleotide primers to levels detectable by hybridization. This reaction is based on the selection of a short DNA segment flanked by two primers each on opposite strands of the segment. These primers define the length of the amplified fragment which is synthesized as follows. First, nucleic acids contained in the sample are heat-denatured. Next, the temperature is lowered to permit reannealing of denatured target DNA with both primers. After completion of this step, the temperature is increased to the optimum for activity of the DNA polymerase, to initiate the synthesis of new strands of DNA complementary to the paired strands. Those are extended from the DNA-primer hybrids by the DNA polymerase. After DNA synthesis, this cycle of DNA dissociation, primer reannealing and DNA elongation is repeated. This technique, first applied for the diagnosis of genetic disease, has also been used for basic research purposes and infectious diseases diagnosis.[37–41]

Application of PCR for HIV-1

Detection *in vitro* of 1 to 30 copies of HIV-1 DNA can be accomplished with PCR (Fig.1 and[23,42–50]) PCR successfully identified HIV-1 DNA in established infected cell lines, cells cultivated from AIDS and AIDS related complex (ARC) patients.[42] It was more sensitive than Southern blot for detection of HIV-1 in viral cocultures.[42] PCR was also applied for detection of provirus in PBMCs of asymptomatic, ARC and AIDS patients.[23,43] PCR was more sensitive than viral culture in asymptomatic individuals from which HIV was cultivated in less than 50% of patients. When first tested, PCR was positive in all seropositive patients with positive cultures for HIV-1 but not in all culture-negative patients.[23] A combination of multiple primer sets could further increase the sensitivity of PCR in this population of patients.[23] Large scale studies conducted afterwards on the use of PCR for HIV-1 diagnosis using the same set of primers demonstrated a high degree of concordance between PCR and serology testings for HIV-1 antibodies.[51,52] In the latter two studies, sensitivity of PCR for detection of infected individuals was over 97%. The lower sensitivity of earlier studies using the same reagents could be explained by the use in the reaction buffer of 50 mM NaCl instead of KCl (see below, section on technical aspects of PCR). PCR was 100% specific with a set of primers for gag, when indeterminate

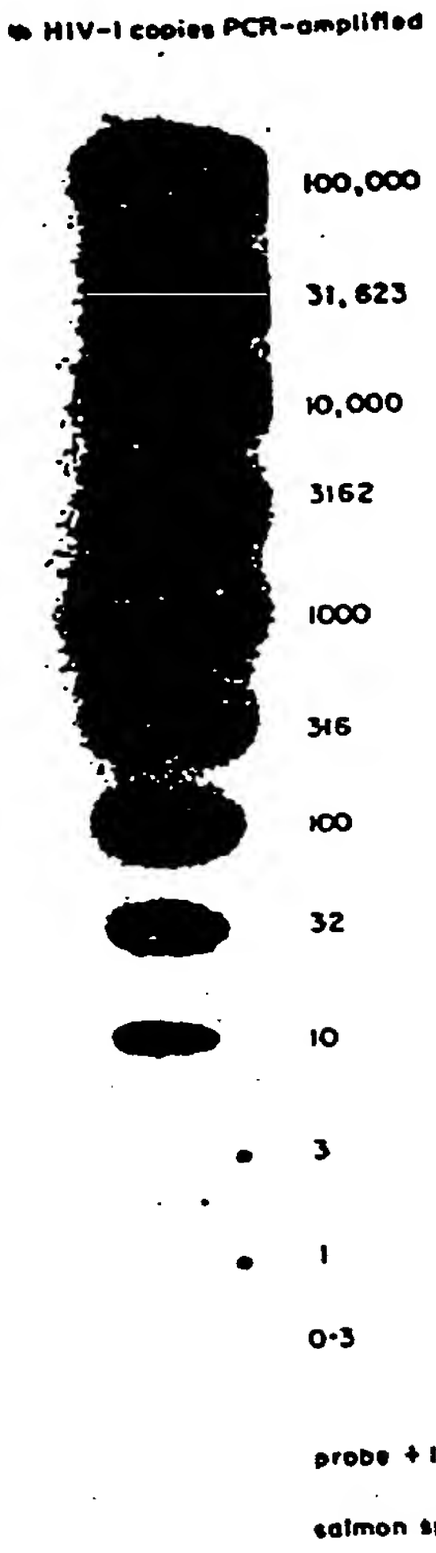

Fig. 1. Sensitivity of PCR for detection of HIV-1 proviral DNA. Serial dilutions in TE buffer of a plasmid containing HIV-1 DNA were amplified with a set of primers for gag gene O-1 and O-2[41,44] through 30 cycles of PCR. Amplified material was spotted on a nylon filter and hybridized with an internal ^{32}P-labelled RNA probe. The autoradiogram is shown here with the number of HIV-1 copies initially amplified. A signal was visible down to one copy (faint signal). Probe is the reactivity of the RNA probe with the products of amplification of 100 ng of salmon sperm DNA.

results were retested (see below).[51] PCR assays can be highly specific with carefully selected primers.[53] The testing of haemophiliac patients provided similar results.[52,54] The use of PCR in those studies helped to establish the universal presence of HIV-1 genome in seropositive patients whether symptomatic or not.

Taq polymerase is specific for DNA substrates.[36,55] By detecting proviral DNA, PCR indicates the presence of at least a latent infection with HIV-1. The detection of HIV-1 RNA is accomplished by including a step before PCR with reverse transcriptase (RT-PCR) to synthesize DNA from HIV-1 RNA contained in samples.[56–60] The presence of replicating virus or of specific mRNA transcripts from HIV-1 can be ascertained with RT-PCR, which helps to further define whether an infection is latent or active. Investigators can use the PCR buffer for the reverse transcriptase step.[61] RT-PCR was 10^4 times more sensitive than detection of unamplified material using a filter-based assay.[57] Crude methods can be used to obtain RNA for RT-PCR.[62] The presence of HIV-1 RNA in the plasma of infected individuals was found to be an early event of primary infection and could be due to cell lysis secondary to cytopathicity and virus production.[59]

TECHNICAL ASPECTS OF PCR

Sample processing

The requirement to process small quantities of blood for PCR assays, without altering its extreme sensitivity, is appealing for clinical diagnosis. Approximately 200,000 PBMC (equivalent to ~1 µg of extracted DNA) are sufficient for PCR analysis. The avoidance of extensive sample treatment and nucleic acid extraction procedures minimizes the risk of false-positive results by carry-over or contamination. PBMCs are purified with a Ficoll Hypaque gradient. Preservation of intact cells can be done in RPMI, 50% fetal calf serum and DMSO at −70°C or in liquid nitrogen.[63] Cells can also be immediately lysed with non-anionic detergents (NP-40, Tween 20) and proteins digested with proteinase K. Heat inactivation of the latter at 95°C for 10 min also introduces additional safety in the handling of sample by further inactivating HIV-1 virions. However, some inhibitors of *Taq* polymerase contained in samples can reduce the efficiency of amplification. Haemin has been recognized as one potential inhibitor and could play an important role if red blood cell lysis has occurred before treatment of specimens.[63] In these situations or when other body fluids are analysed such as stool samples[64] or breast milk (F. Coutlée, pers. comm.), more extensive purification procedures may be needed. To avoid contamination of PCR with inhibitors contained in blood which is not freshly processed, a protocol consisting of lysis of red cells and repetitive washing steps to eliminate haemin has been suggested.[63] Phenol/chloroform extractions followed by ethanol precipitation can further purify these samples. PCR inhibitors are further discussed in section 'disadvantages of PCR'. Processed samples can be stored at −70°C.

DNA extracted from formalin-fixed, paraffin-embedded tissues from lymph nodes, brain, lung and skin have been demonstrated to be suitable to amplification with PCR.[65,66,67]

Choice of primers

To ensure efficient amplification of wild type strains of HIV-1, highly conserved regions of the genome are selected. Equally important is the selection of regions with little cross-reactivity with other retroviruses, to obtain specific results. Multiple primer pairs have been tested with clinical specimens (Table 1). Reagents for gag gene included sets SK39/SK38,[23,42,51,52,54] GK55/GK56,[72] 0-1/0-2[45] and SK101/145.[51,68,69] The latter pair was as sensitive as SK38/39 and amplifies a 130 bp region of gag which is conserved among HIV-1 and HIV-2 variants. SK145 can be replaced by SK150. Primer sequences have also been directed at the LTR region with SK29/SK30,[23] P13/P14;[53] envelope gene with SK69/SK68,[23] CO1/CO2,[23] K4/K5,[71] CO71/CO72,[54] 88-79/88-272[71] and *tat* with CO11/CO12.[60] Sets for amplification of envelope segments could sometimes detect HIV-1 in specimens negative with SK38/SK39.[23] However, some investigators did experience specificity problems with amplification of envelope segments of HIV-1.[47] General rules can guide those who desire to design their own amplimers.[73,74] They are usually 18 to 40 bases long. One should avoid complementarity at the 3′ end of primers as well as GC rich 3′ ends to minimize primer–dimer formation. Primers should not span palindromic sequences. Primers with too high G+C contents can theoretically bind non-specifically to DNA and generate aberrant reactions.

Parameters of the amplification reaction

Optimization of parameters for amplification of HIV-1 and HIV-2 viruses have been systematically studied.[74,75] Such analysis should be repeated for each new pair of primers used. The conditions under which amplification occurs influence greatly the specificity

Table 1 Primers for HIV-1 detection

Primer	Primer sequence	Region	Fragment(bp)
SK38	ATAACCACCTATCCCAGTAGGAGAAAT	gag	115(HIV1)
SK39	TTTGGTCCTTGTCTTATGTCCAGAATGC		
SK19(P)	ATCCTGGGATTAAATAAAATAGTAAGAATGTATAGCCCTAC		
SK145	AGTGGGGGGACATCAAGCAGCCCATGCAAAT	gag	141(HIV1) 139(HIV2)
SK101	GCTATGTCAGTTCCCCTTGGTTCTC		
SK102(P)	GAGACCATCAATGAGGAAGCTGCAGAATGGGAT		
SK145	AGTGGGGGGACATCAAGCAGCCCATGCAAAT	gag	142(HIV1) 139(HIV2)
SK150	TGCTATGTCACTTCCCCTTGGTTCTCTC		
SK102(P)	GAGACCATCAATGAGGAAGCTGCAGAATGGGAT		
O-1	GGGCAAATGGTACATCAGGCCATATCAC	gag	525(HIV1)
O-2	TTTTACCTCCTGTGAAGCTTGCTCGGTC	gag	422(HIV1)
I-2	TTGGTCCTTGTCTTATGTCC		
I-1	TTAATACGACTCACTATAGGGTAGAAGAGAAGGCTTTCAGC	gag	190(HIV1)
GK55	CCTGCTATGTCACTTCCCCT	pol	355(HIV1)
GK56	TTATCAGAAGGAGCCACCCC		
P-1	TTCTGTCAATGGCCATTGTTTAACTTTTGGGCCAT	ltr	105(HIV1)
P-2	TAAAGGAAGCAAGCTCTATTAGATACAGGAGCAGCTGA		
SK29	ACTAGGGAACCCACTGCT	LTR	168
SK30	GGTCTGAGGGATCTCTA		
SK31(P)	ACCAGAGTCACACAACAGACGGGCACACACTACT		
P13	TGGCGCCCGAACAGGGAC	LTR	165(HIV2)
P14	TACCCACGCTCTCGCAG		
SK89	ACGAGCTGGTGGGGAACG	env	142(HIV1)
SK90	GTGCTGGTGAGAGTCTAGCA		
SK91(P)	TTGAGCCCTGGGAGGTTCTCTCCAGCACTAGCAGGTAG		
SK68	AGCAGCAGGAAGCACTATGG	env	135(HIV1)
SK69	CCAGACTGTGAGTTGCAACAG		
SK70(P)	ACGGTACAGGCCAGACAATTATTGTCTGGTATAGT		
CO1	ACAATTATTGTCTGGTATAG	env	320(HIV1)
CO2	AGGTATCTTTCCACAGCCAG		
CO3(P)	TGAGTTGCAACAGATGCTGTTGCGCCTCAATAGCCCTCAG		
CO71	TGTGGAGGGAATTCTTCTACTGTAA		
CO72	TATAGAATTCACTTCTCCAATTGTCCCTCAT		
CO75(P)	CAAATATTACAGGGCTGCTATTAACAAGAGATGGTGGTAA		

and extent of DNA synthesis. Those conditions are reviewed here step by step.

Denaturation of processed samples before the performance of the first step of PCR is advisable especially for detection of sequences integrated in the cellular genome. A thorough separation of long strands of DNA increases the accessibility of primers to target sequences. Samples can then be added to the PCR reaction mixture. The denaturation temperature for the first step of each PCR cycle should not exceed 95°C to circumvent repetitive heat-inactivation of *Taq* by heat.[73]

In the second step of a cycle, reannealing of primers to target DNA will depend on the ionic strength of buffer, temperature of the reaction, hybridization time and primer concentration.[74,75] To facilitate reassociation of primers to target sequences, up to 50 mM of KCl is included in the reaction buffer. A higher concentration of KCl or the replacement of KCl by NaCl will inhibit the amplification reaction.[55,74,76,80] The temperature at which this step should be done can be calculated at Tm − 5°C.[77] Lower temperatures will allow non-specific binding of primers and generate multiple non-specific by-products with a lower specific signal.[36,74,75] In fact, non-specific amplification occurs when one step of the cycle is carried under low stringency conditions even though the other steps are performed at high temperatures.[74] Reannealing carried for 30 s can amplify HIV-1 but is less efficient than a 60-s period.[74,75] Since too high or too low concentrations of primers reduce the efficiency of amplification, from 0·1 to 0·5 μM of each primer should be added to the reaction buffer.[73–75,79] High primer concentrations result in mispriming and

self-hybridization with primer–dimer formation, which both compete for reagents with the specific segment and can lead to competitive inhibition.

Three enzymes have been used in PCR to synthesize specific DNA segments: the Klenow fragment of *E. coli* DNA polymerase I[34,37,42] *Taq* DNA polymerase[23,36] and T4 DNA polymerase.[78] Klenow is heat-labile and reaction mixtures need to be supplemented in enzyme after each denaturation step. *Taq* is a thermostable DNA polymerase from *Thermus aquaticus*.[55] The use of *Taq* has simplified PCR protocols, since it does not need to be replenished after each denaturation step. The amplification procedure can be automated. *Taq* can amplify fragments of at least 2 kb with good fidelity.[36] The saturation plateau of amplification is reached more rapidly with Klenow than with *Taq*.[36] Because *Taq* tolerates higher temperatures, the specificity of amplification is improved. Consumption of reagents in amplification of non-specific products is reduced and a better sensitivity level is reached.[36] Increased specific and non-specific amplifications were observed with higher quantities of *Taq* and longer extension times.[36] The addition of enzyme at 10 and 20 cycles can result in the generation of non-specific bands, while addition of *Taq* at 15 cycles can lead to a sharper specific band.[74] *Taq* from different commercial sources exhibited differences up to 200-fold in efficiency.[75] The composition of the reaction mixture greatly influences the efficacy of *Taq*. The activity of this enzyme is magnesium and pH-dependant.

The concentration of $MgCl_2$ is the most critical component of the buffer to optimize. Activity and fidelity of this enzyme are dependent on free Mg^{2+} concentration. Too high a concentration of Mg^{2+} inhibits amplification.[54,73,75,80] Some investigators have observed that a high requirement in $MgCl_2$ could be indicative of loss activity of the enzyme batch.[75] The pH of the reaction buffer should be kept at least at 8·0, preferably from 8·3 to 8·8 (at room temperature).[55,75,80] It should be supplemented with dNTPs at concentrations from 20–200 μM, in equal amounts to minimize misincorporation and mispriming.[73,74,76] DMSO 10% should not be included in the reaction buffer since it could be inhibitory.[73] Gelatin is also included in the reaction buffer to stabilize the enzyme although too much is inhibitory.[80]

The efficiency and reproducibility of PCR is increased with addition of mineral oil on top of the reaction volume in order to avoid variation in reagents concentration due to evaporation during the different cycles.[81]

The PCR procedure can be shortened by reannealing amplimers to target and extending with DNA polymerase at 70°C, but little amplification was found under these conditions.[74] With some primer sets, reannealing and elongation can be done at 55°C or 60°C.[74,75] Variations of elongation temperature between 50–70°C did not influence drastically amplification of pure target. Extension times of 1 min are adequate. Longer segments to amplify require longer elongation time.[75]

A greater number of cycles with PCR results in increased amplification.[36,74] Theoretically, amplified products should accumulate exponentially.[36,83] However, a reduction in efficiency of PCR after 20 cycles has been observed.[74] A plateau is reached at 30 cycles which is only partially corrected by the addition of *Taq* after 20 cycles.[36,75] The efficiency of PCR rapidly drops with high initial amounts of template.[48,82] The reduced efficiency of PCR over time can be explained by partial inactivation of *Taq* by repeated heat denaturation, consumption of deoxynucleotides and primers, and competition between specific and non-specific amplified DNA.

Detection methods of amplified products

The most convenient method for detection of specific products of amplification consists in the electrophoresis of amplified products in agarose or polyacrylamide gels and visualization of specific bands with ethidium bromide staining (Fig. 2 and [36,45,46,83]) The precise determination of size of amplified DNA segments can be accomplished in less than 1 h. However, hybridization reactions are more sensitive for the detection of PCR-amplified products.[36,45,46,70] Hybridization reactions also introduced an additional step of specificity, providing more confidence in positive results.[85] Although reamplification with the same set of primers does not consistently result in increased sensitivity of gel electrophoresis,[45] reamplification of PCR products with a nested set of primers reached a level of sensitivity equivalent to levels obtained with hybridization methods after one round of PCR.[84] Although the second round of PCR adds sensitivity and specificity to the assay, this latter method suffers from the inconvenience and cost of performing two PCR reactions.

Detection of amplified DNA can be accomplished using homogeneous or heterogeneous hybridization reactions. In the latter assays, a specific probe is reannealed with amplified DNA sequences immobilized on a solid support. Usually amplified products are hybridized with a ^{32}P end-labelled oligonucleotide probe and detected with autoradiography. The sequences of the oligonucleotide are chosen within the amplified segment without overlapping with the

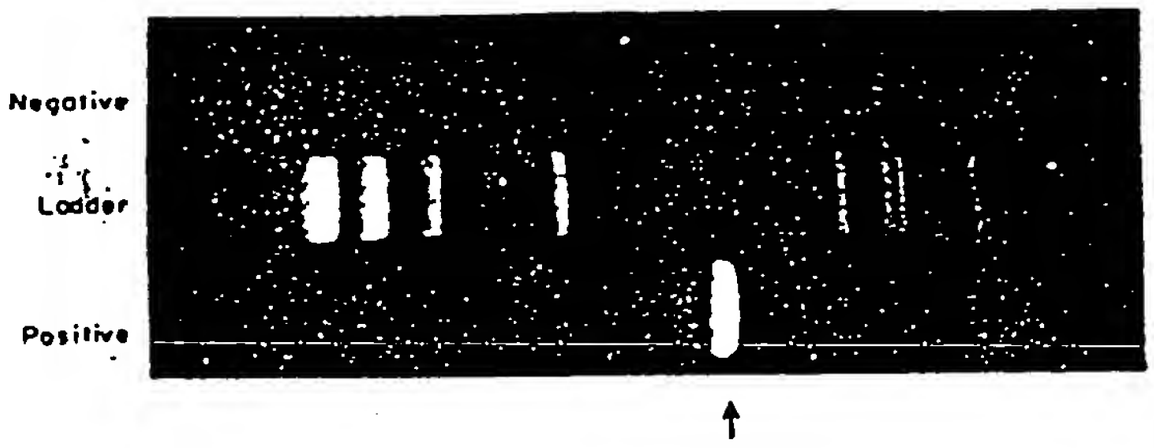

Fig. 2. Dectection of PCR products with gel electrophoresis. One pg of a plasmid containing HIV-1 DNA was amplified with primers I-1 and I-2 generating a 422 bp fragment from *gag* gene.[45,46] 10 µl of the reaction mixture was electrophoresed in a 3% NuSieve-1% agarose gel stained with 0·5 µg ml^{-1} of ethidium bromide. Line POS is the amplification of the HIV-1 plasmid and line NEG is amplification of buffer alone. LADDER is a molecular size ladder of *Hae* III-digested phiX174. The arrow indicates the location of the specific band.

primers. After gel electrophoresis, amplified DNA can be transferred onto filters by Southern transfer.[36,85] The specificity of a positive reaction is then controlled at two levels: size of amplified product and reactivity with a specific internal oligonucleotide probe. End-products of PCR can also be applied directly onto membranes as in dot- or slot-blots techniques.[85] To increase the overall sensitivity of PCR, transcriptional enhancement of amplified products has been described.[58] Sequences from the T7 RNA polymerase promoter are appended at the 5′ end of one of the primers. Specific amplified fragments thus incorporate the double-stranded T7 promoter at their 5′ end which allows transcription of amplified product with T7 RNA polymerase. Alternative techniques for detection of amplified products have been explored to circumvent variability of binding of nucleic acids to filters and time required for completion of filter-based hybridization reactions.

In homogeneous methods, a probe is reacted in solution with PCR-amplified products. Specific amplified fragment-probe hybrids can be identified by electrophoresis in gels. One of these methods, the oligomer restriction technique,[39-42] involved hybridization of amplified products to an internal ^{32}P-labelled oligonucleotide containing sequences for a restriction enzyme site. After hybridization, the specific double-stranded endonuclease restriction site was reconstituted. With the addition of the appropriate enzyme, cleavage of hybrids generated a shorter DNA–DNA duplex which could be separated from unhybridized probe by gel electrophoresis. Following autoradiography of the gel, the presence of the band of defined size was considered diagnostic. Concerns of loss of sensitivity due to single-base substitutions and unpredictability of restriction enzyme reactions in PCR buffer limit the applicability of the oligomer restriction method.[85] The probe-shift assay[47] or oligomer hybridization (Fig. 3 and [51,70]) is a faster variant of this procedure. Hybrids between amplified products and the ^{32}P-oligonucleotide probe are fractionated from unreacted probe on a non-denaturing gel without predigestion with a restriction enzyme. Since analysis of amplified products is dependent upon rate of migration, a control amplification reaction with K-ras can be run concomitantly in the same gel.[47]

Another method for identification of amplified products consists in the incorporation of a radiolabelled dNTP during PCR and gel electrophoresis of amplified products.[70] However, radiolabelled dNTPs can be incorporated into any elongating DNA chain. Moreover, non-specific bands can sometimes comigrate at the same level as the specific band.[85] In those situations only a specific internal probe can identify a specific from a non-specific reaction. The affinity-based hybrid collection method, proposed by Harju *et al.*, is a convenient assay to test a great number of specimens and is also sensitive.[48] PCR is performed as usual but with 5′-biotinylated primers. Biotinylated DNA is then reacted with a ^{35}S-labelled probe. Hybrids are captured on avidin-coated particles, washed and measured by Cherenkov counting.

All these assays suffer from the drawback of using radiolabelled probes which have a short functional half-life for identification of PCR products. Non-isotopic means for detection of PCR-amplified products would be desirable to eliminate the biohazards, expense and disposals problems imposed by radioactivity. One proposed system was based on the colorimetric detection of amplified DNA by incorporation, in the last few cycles of PCR, of a nested set of oligonucleotides, each labelled with a different ligand.[87] One nested primer was biotinylated and the other contains a DNA binding protein site for capture. This system is, however, not completely free from false-positive results due to mispriming. Filter-based assays using oligonucleotides covalently linked to alkaline phosphatase, have also been described for

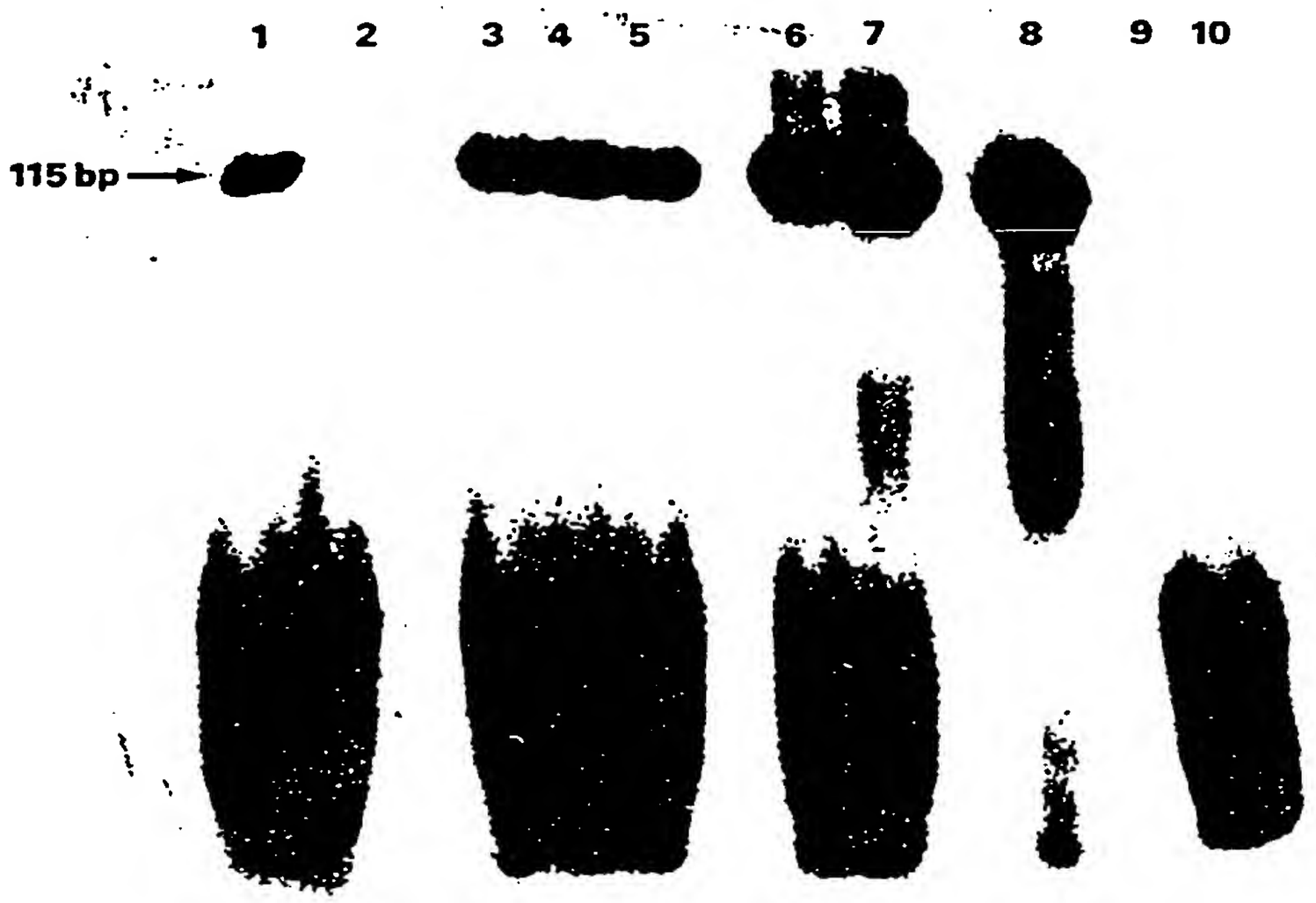

Fig. 3. Probe-shift assay. PBMCs were amplified through 30 cycles of PCR with primers SK38/SK39. One-tenth of the amplified products was hybridized in 0·15 M NaCl to SK19 end-labelled with ^{32}P for 30 min at 55°C. The reaction mixtures were then fractionated on a 12% polyacrylamide gel. The autoradiogram is shown here. PROBE is the location of unhybridized probe. Location of the 115 bp fragment is indicated on the left of the figure. Lane 1 is from a seropositive control; lane 2 from a healthy seronegative control; lanes 3, 4 and 5 are triplicate testings from an asymptomatic seropositive patient; lanes 6 and 7 are duplicates from an AIDS patient; lane 8 is also from an AIDS patient; lane 9 was a molecular size ladder visualized with ethidium bromide staining (not shown); lane 10 is a buffer control.

genetic purposes and HIV-1 detection.[89,90] PCR combined with a microtitre plate sandwich hybridization assay using biotin-labelled probes could detect 30 molecules of HIV-1.[72]

A promising homogeneous assay used an internal oligonucleotide probe labelled with a chemiluminescent acridinium ester.[49,50,88] It can be completed in less than 30 min. The test is based on the selective chemical hydrolysis of unreacted probe which does not affect the signal from the specifically hybridized probe. Specific hybrids are measured in a luminometer. Another strategy combined the hybridization of amplified DNA and specific RNA probes with the immunological detection of specific hybrids in a standard enzyme immunoassay format.[45,46] Unlabelled or biotinylated RNA probes are hybridized in solution with amplified DNA. The DNA–RNA hybrids are then captured onto wells of a microtitre plate coated with an antibody to biotin for biotin-labelled probes or with a monoclonal antibody against DNA–RNA duplexes for unlabelled probes. Hybrids bound to the solid phase are detected by reactivity with a monoclonal antibody to DNA–RNA hybrids labelled with an enzyme. Detection of the enzyme labelled antibody can be accomplished with fluorogenic or colorigenic substrates.[90a] The assay could be completed in less than 4 hours and could detect 10 proviral copies (Fig. 4). The monoclonal antibody to DNA–RNA hybrids produced by R. J. Carrico from Miles laboratories recognizes the conformation of DNA–RNA hybrids without reacting with double-stranded DNA or single-stranded RNA or DNA. A nested set of primers is used in a separate reaction to generate an inner fragment composed of sequences between the outer set of primers. Since one of the amplimer has sequences of the T7 RNA polymerase promoter appended to its 5′ end, a specific single-stranded RNA probe can be transcribed and used for detection of amplified HIV-1 segment.

Criteria for positivity for PCR

Criteria for positivity for PCR have been proposed as follows.[51,52,69,106] For a single set of primers, a sample is considered positive if duplicate testings of that

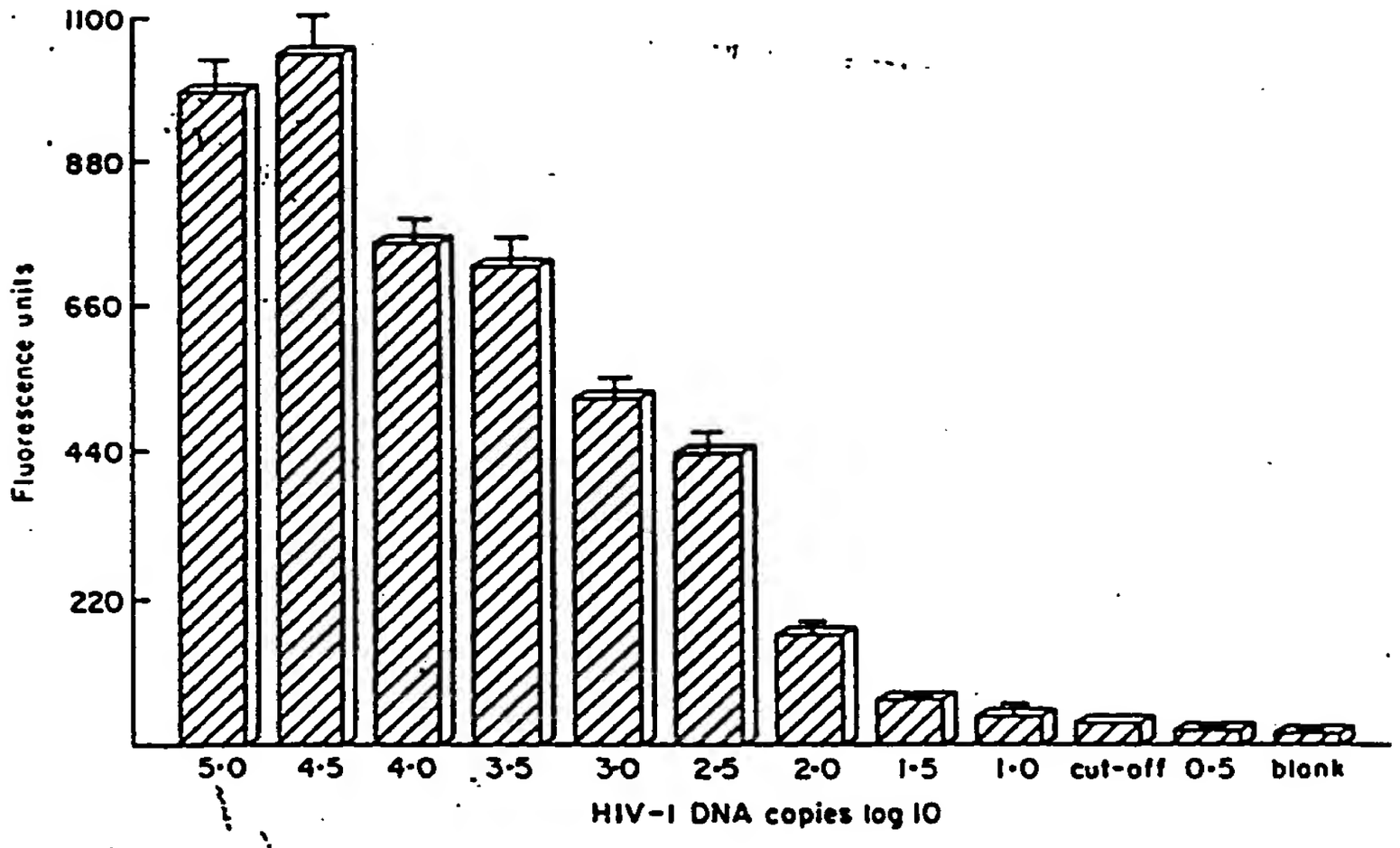

Fig. 4. Non-isotopic detection of HIV-1 DNA with PCR-EIA. Dilutions of a plasmid containing HIV-1 DNA were amplified with the set of primers O-1 and O-2 under conditions described in reference 45. An enzyme immunoassay (EIA) for DNA-RNA hybrids was done after hybridization of amplified material with an internal unlabelled RNA probe. The reactivity in the EIA was measured in fluorescent units (Y-axis). The cut-off was the mean reactivity of three determinations of the background noise of the RNA probe with a lysate of 10^4 Vero cells amplified with O-1 O-2 (blank), plus 3 standard deviations.[45]

sample are clearly reactive, is negative if both duplicates fail to be reactive. The presence of specific reactivity in only one of the duplicate testings is classified as indeterminate. Specimens are considered positive if at least one set of primers amplified specific sequences for HIV-1, negative if all amplification reactions were negative and indeterminate in all other situations. Indeterminate samples warrant the testing of additional samples from the same patient.

Advantages of PCR

Since amplification is partially automated, PCR requires shorter hands-on time and faster turnaround time than culture. The assay is exquisitely sensitive and specific with the appropriate selection of primers. It uses a small sample volume. This technique allows for the detection of active (HIV-1 RNA) as well as latent infections, without dependance on the host immunological response or on the expression of proteins by HIV-1. Multiple primer pairs to different areas of the HIV-1 genome and to other viruses such as HTLV-1 could be mixed in the same reaction tube.[91] The impact of this mixture of reagents on the sensitivity of amplification needs, however, to be addressed. Finally, amplified material is non-infectious as opposed to viral culture.

Disadvantages of PCR

The extreme sensitivity of PCR by itself leads to the most problematic disadvantage of this technique: the increased likelihood of detection of trace amounts of contamination.[92,93] Sources for contaminating PCR reactions include carry-over between samples, especially if aerosols are generated, shedding of dead epidermal cells, plasmid vectors containing HIV inserts, or strong positive controls.[94,95] Preventive measures can reduce the occurrence of contamination.[68,83,94,95] In our experience, the most critical measures to reduce contamination are the physical containment of pre-PCR manipulations from post-PCR products, the use of dedicated pipettor for PCR and of disposable pipettes for blood processing, the avoidance of recombinant plasmids as positive controls, the use of positive controls at low titres, the aliquoting of PCR reagents, and the avoidance of aerosols during the manipulation of samples. However, sources of contamination are not always obvious. Inclusion of numerous patient and buffer controls permits early recognition of contamination. Duplicate testings of samples can also prevent reporting false-positive results.[51] Keeping aside an aliquot of each sample allows the re-testing of samples when contamination has occurred with the first aliquot.

One problem also encountered by multiple investigators has been the inconsistent detection of HIV-1 sequences with different pairs of primers. A loss of integrity of DNA during sample manipulation could explain some false-negative results. The co-amplification with primers for cellular genes HLA-DQ alpha,[51,52] K-ras[47] or B-globin,[50] can control for the integrity and amount of DNA input in the reaction. The presence of secondary DNA structure in target DNA could be a cause of false-negative results. Inhibitors of *Taq* polymerase co-purifying with DNA could play an important role in cases of seropositive patients negative with PCR.

Such inhibitors have been described in blood, stool and urine samples.[48,63,64] The importance of sample purity was demonstrated in one study were lymphocytes were prepared under difficult conditions.[96] Extensive extraction procedures were then required in order to amplify control cellular DNA. Some inhibitors could only be removed from DNA by boiling and gel filtration.[97] Amplification of a cellular gene could identify those samples that contain inhibitors of PCR. However, it has not been firmly established that the degree of inhibition of amplification is uniform for different sets of primers. Also, the degree of inhibition of PCR for low copy number targets could be difficult to evaluate with controls detecting abundant sequences that reach saturation of the amplification process early in PCR.

A low viral load could also explain non-reproducible results. In borderline cases, interference of specific amplification by non-specific reactions or suboptimal amplification conditions could cause false-negative reactions. One problem may well be the variable efficiency of primers or probe to bind to target DNA from diverse strains. Highly conserved areas have been identified in the HIV-1 genome in LTR and *gag* genes, while other regions exhibit strain to strain variations[98-101] which may incapacitate hybridization assays. Sequence alterations can affect primer reannealing and inhibit the amplification reaction. A second set of primer directed to another region of the genome enabled some groups to reduce the occurrence of false-negative PCRs.[23,96,102] The effect of location and type of mismatches between target and primers have been investigated in details.[103]

Mismatches located at the 3′ end of primers are the most likely to inhibit the amplification process. In contrast to Klenow, *Taq* lacks a 3′–5′ exonuclease activity[55] and cannot correct mispaired bases at the 3′ end. Kwok *et al.* found that the presence of a T at the 3′ end of primers was efficient for PCR, irrespective of the nucleotide at that position on the template. However, the following mismatches between template and primers (A:C, C:A and C:C) generated a 100-fold reduction in efficiency of amplification. The effects of mismatches were symmetrical. A A:A mismatch moderately reduced PCR efficiency. The most significant inhibition occurred with purine–purine mismatches. A lower concentration of dNTPs accentuated the inhibitory effect on PCR of mismatched ends. Internal single and multiple mismatches more than eight bases from the 3′ end did not alter significantly the efficiency of PCR. Two mismatches in the last four bases of a primer (but not involving the last base at the 3′ end) reduced PCR efficiency while only one did not. That study concluded that a single or double T at the 3′ end of primers would benefit assays directed at the detection of agents with genomic variation. 3′-inosine substituted primers could also be used successfully to prevent failures of PCR due to 3′ end mismatches.[104,105]

CLINICAL APPLICATIONS OF PCR

PCR has not been demonstrated yet to be a suitable screening tool for HIV-1. In large scale studies, a good concordance was found between serological assays and PCR. Serology assays are thus sufficient to identify infected individuals in a screening setting. However, PCR could become indicated in selected situations.

PCR for seronegative individuals at risk

Patients before and after seroconversion were found to carry HIV-1 DNA and HIV-1 RNA with PCR.[59] Several publications have suggested that high-risk individuals can harbour HIV-1 DNA up to 3 years prior to the development of antibodies to structural proteins of HIV-1.[44,106,107,109,110] This could raise concerns about the validity of routine blood screening methods currently available. However, this phenomenon seems to be quite rare, since large surveys of seronegative at risk individuals from other investigators have failed to detect it.[51,52,54,111,112] These last studies may have been partly biased by inclusion of patients less likely to be recently infected with HIV-1.[51] Seronegative individuals positive for HIV-1 DNA by PCR were recruited mostly from high risk homosexuals or heterosexual partners of symptomatic and asymptomatic seropositive patients,[106,109,110,113-115] but also from infants born to seropositive mothers[102] and from intravenous drug addicts.[110,113] High risk individuals in most studies were defined as those using intravenous drugs and sharing needles with a seropositive patient or those having unprotected sex with

a seropositive partner. Small numbers of PCR positive-antibody negative individuals have been described and the exact prevalence rate is unknown. Some patients are positive using two sets of primers for as long as 12 months.[44,109,114] Of 133 seronegative homosexuals at high risk, 27 (20%) had positive HIV culture and/or PCR for HIV-1, but remained seronegative for up to 3 years.[106] Ameisan *et al.*[109] explored the possibility that these patients were seroconverting to regulatory proteins before seroconverting to structural proteins. Seropositive patients for anti-nef antibodies were identified in this subgroup of patients. Transmission of HIV-1 from a seronegative patient to his sexual partners even occurred.[115]

Multiple hypothesis have been considered to explain this phenomenon.[107] Is there a defective form of HIV-1 which is unable to synthesize structural proteins and is thus incapable of replication? Could some patients eliminate HIV before seroconversion? However, some patients had positive cultures. Contamination of PCR could explain some of these results which could be false-positive PCR assays. But confirmation of the presence of HIV-1 with other techniques validates some of these studies.[19,106] More extensive studies including large numbers of at high risk individuals are needed to determine the importance of this prolonged phase of immunological silence and to explain the underlying mechanisms. The only study which found rare seropositive patients who seroreverted but remained PCR positive for HIV-1 needs confirmation.[117]

Pediatric AIDS

Early diagnosis of HIV-1 infection in infants is important since most of the perinatally infected patients develop disease in their first year of age.[118] The short incubation time of AIDS in infants, the need to avoid toxic treatments when unnecessary, and the identification of at risk patients for opportunistic infections, all support the necessity for a precise means of detecting HIV-1 in infants. PCR could help determine the time of acquisition of the virus (intrauterine or perinatal) in research studies, which would help to define recommendations to reduce the risk of transmission of this agent to infants. The knowledge of infection status could also alleviate some parental anxiety in uninfected infants. Serology testings cannot be interpreted until 15 months of age since transplacental passage of antibody can occur in the absence of infection. Because of the small volume of cord blood available, lymphocyte cultures are difficult to perform. The antigen detection test is not sensitive enough to identify all infected infants.

With PCR, HIV-1 genome was identified in PBMCs from cord blood or blood samples of 33–55% of infants and children born to seropositive mothers.[45,102,119–123] Previous studies had reported similar estimates of mother to infant transmission of HIV-1.[124] PCR required only 0·2 ml of blood.[120] Cord blood could be contaminated at birth with maternal mononuclear cells resulting in a false-positive result with PCR. Further testing of positive patients can eliminate this possibility. Nearly all the symptomatic patients were positive with PCR in those studies. Some false-negative results in infants tested only at birth could have been related to insufficient proviral copy numbers in the sample. PCR identified most infants who further developed AIDS or symptoms suggestive of HIV infection.[119,120,123] HIV was also found in patients with non-specific clinical findings. Prospective assessment of these children with mild clinical features will determine how many will develop AIDS. In multiple studies, negative results for HIV-1 with PCR in the postnatal period indicated an absence of infection in the presence of maternal antibodies.[119–121,123] Prospective evaluation of patients with indeterminate PCR results helped clarify their HIV status,[119] emphasizing the importance of interpreting with caution weak positive results. In Africa, a higher transmission rate of HIV-1 from mother to infants of 64% was demonstrated with PCR.[96] This high rate dropped to 45% when only samples reactive with two sets of primers were considered positive. PCR supported the occurrence of in utero transmission of HIV although some patients seemed to acquire the virus afer the neonatal period.[120]

PCR also allowed the diagnosis of infected children who had lost maternal antibodies against HIV.[102,119] The synthesis of specific anti-HIV antibodies by the children may require 12 months after the disappearance of maternal antibodies.[125]

Evaluation of neurological infection

The HIV-1 genome has been identified using PCR in brain tissue.[126] PCR has also demonstrated the presence of HIV-1 DNA in paraffin-embedded tissue from the brain in a case of fatal acute encephalitis without other etiology.[67] Proviral DNA was also detected in mononuclear cells from the cerebrospinal fluid (CSF) from almost all patients who had neurological abnormalities and CSF pleocytosis clinically attributed to HIV-1.[108] Those with negative CSF for HIV-1 did not develop CNS pathology.

Presence of HIV-1 in human body fluids

The presence of HIV-1 viraemia has been substantiated by Hewlett *et al.* by demonstrating the presence of HIV-1 RNA and DNA in plasma.[59] Semen mononuclear cells and semen supernatants from HIV-1 infected symptomatic patients[42] were also found to contain proviral DNA. Also, PCR identified the presence of HIV-1 DNA in exudates from genital ulcers of seropositive men.[127]

One limitation of PCR is the inability to identify the cell types which harbour the amplified sequences. One author reported the successful combination of PCR with *in situ* hybridization.[128] In his technique, fixed cells were rendered permeable to allow intracellular penetration of PCR reagents, including *Taq* polymerase. Amplification was performed in cells which were probed immediately after PCR to limit diffusion of amplified DNA outside the cell. This modification of PCR was applied to HIV infected cell lines and sperm cells with success.

Discrimination of dual infections with related retroviruses

PCR has also been useful for confirmation of co-infection with HIV-1/HIV-2[129] and HIV-1/HTLV-1[130] in cases with dual reactive serologies.

Resolution of indeterminate Western blot

Some patients have reactive EIAs for HIV antibodies with indeterminate Western blots. PCR has demonstrated that individuals at low risk with indeterminate serologies for HIV-1 are not infected with HIV-1.[69,131] PCR could help resolve cases with indeterminate serology results.

Drug trials

If PCR could be used quantitatively, the evolution of proviral load could help to evaluate the response to treatment.[132,133] The presence of viral RNA is indicative of active replication and could allow detection of reactivation in patients under treatment.[71] However, in one study, no reduction in viral copy number of RNA over a 1-year period was observed with the administration of anti-retroviral drugs, a finding which was attributed to variations in the PCR procedure.[71]

Other areas of interest

Since PCR allows detection of DNA without relying on the presence or absence of host antibodies, this technique would be useful to follow vaccinated patients in trials or after exposures at risk. If antiviral therapy would prove useful after a puncture wound with contaminated biological fluids, a follow-up with PCR would detect an early infection and guide clinicians for therapy. Finally, PCR has been used to detect potential cofactors that might be associated with HIV in the pathogenesis of AIDS.[134]

APPLICATIONS OF PCR FOR RESEARCH PURPOSES

Rapid probe preparation

DNA or RNA probes can be prepared with the help of PCR, by amplification of insert sequences with primers flanking vector sequences.[135] The probes can also be labelled during the amplification process.[136] Another strategy consists in generating a DNA fragment with incorporation of the T7 RNA polymerase promoter in the amplified DNA segment.[45,46,136] PCR-amplified material is then used as a template in a standard transcription reaction to synthesize specific biotin-labelled or radiolabelled RNA probes. This procedure has been applied to biotin-labelled and radiolabelled probes.[45,136]

Evaluation of target cells for HIV-1 *in vivo*

PCR has been used to study the cell types harbouring HIV-1. Purified subpopulations of PBMCs were subjected to PCR for HIV-1 detection.[137,138] *In vitro* infection of CD4+ cells with HIV-1 results in a down regulation of expression of the CD4 molecule. However, amplification of PBMCs from infected patients demonstrated that *in vivo*, host cells for HIV-1 still expressed the CD4 receptor. CD4+ T cells were the main reservoir for HIV-1 in PBMCs. Few patients had HIV-infected monocytes.

The possibility that HIV-1 could infect eosinophils and the importance of such occurrence is currently under investigation with PCR.[141]

The intracellular molecular structure of proviral DNA has been investigated with PCR since the viral copy number in human tissue is not sufficient to allow such analysis with standard procedures.[139]

Primers were selected to amplify segments of HIV-1 representative of the HIV-1 genome at different steps of reverse transcription and integration into the host chromosome. These studies demonstrated that reverse transcription is initiated as efficiently in resting T cells as in stimulated lymphocytes, but that it is incomplete. An incomplete provirus was thus synthesized in the unstimulated cell and could be degraded in these cells. This process could be responsible for the protracted persistant infection with HIV-1. The same strategy was used on brain material from seropositive patients.[140] After nucleic acid extraction from brain biopsies, integrated and unintegrated species of proviral DNA could be fractionated by electrophoresis into high and low molecular mass DNA. Each fraction was subjected to PCR. High levels of unintegrated forms of proviral DNA correlated with the presence of HIV encephalitis. The unintegrated linear, unintegrated circular and integrated proviral DNA forms were found in brain and blood specimens from AIDS patients. However those with AIDS-related encephalitis contained a higher proportion of unintegrated proviral DNA. The relative amount of HIV-1 DNA was greater in brain specimens from encephalitis patients than in PBMC from other AIDS patients or brains from non-HIV encephalitis.

Evaluation of viral load

Since PCR reaches extreme limits of sensitivity, it could become a useful tool for measuring HIV-1 DNA copy number in PBMCs in relation to antiviral therapy or evolution towards disease. Individuals with progressive disease harbour higher titres of virus and have a greater proportion of infected CD4+ cells.[142,143] Studies using PCR to evaluate quantitatively the proviral copy number in PBMCs found that symptomatic seropositive patients have a larger viral burden (from 1/100 to 1/1000 infected CD4+ cells) than asymptomatic patients (from 1/1000 to 1/40,000).[84,116,137,138] Quantitation in two studies[137,138] was accomplished by comparing signals from amplifications of dilutions of PBMCs with the signals generated by a standard curve from a chronically infected cell line. In one study,[53] the viral copy number was estimated with a slot-blot assay of amplified HIV-1. Even under controlled conditions, variations in efficiency of amplification with PCR generate differences in yields of amplified products.

In one study, PCR was found not to be reproducible enough nor sufficiently precise to allow discrimination of differences in HIV-1 RNA copy number less than five-fold.[71] We have found that the intra-run coefficient of variation of PCR could be as high as 10%.[45] Amplification of plasmid DNA may be more efficient than amplification of genomic sequences.[74] Comparisons of signals from amplified samples with those obtained with a standard curve generated with a cloned plasmid may be inaccurate. Variability in the efficiency of PCR will impose some limitations on the accuracy of viral copy number estimations. Subsaturating number of PCR cycles are required to avoid reaching the plateau of amplification.[53] The number of cycles must be kept in the range where the rate of amplification is exponential. It is also preferable to quantitate amplified products with an homogeneous hybridization assay to avoid variable binding of nucleic acids to solid supports.

In order to attempt to quantitate HIV-1 in samples without dependence on filter-based assays, a PCR assay using a standard curve with incorporation of radiolabelled primer but not an internal standard has been described.[140,145] Control of PCR variability was also attempted by co-amplification of the B-globin gene to quantitate the total amount of cellular DNA input.[140] The authors claimed they could resolve two-fold differences with this technique. However, quantitative use of PCR is difficult because of the dependence of measurement of initial DNA on the efficiency of amplification, which may be variable from sample to sample and between primer sets.[146] A truly quantitative method must also account for factors contained in the sample that influence the amplification process. An internal standard amplified with the same primers used for amplification of target sequences would thus be desirable.

One group developed a quantitative PCR assay for mRNA with internal standards to control for variability of PCR.[146,147] They constructed a synthetic internal standard from which cRNA could be transcribed. The internal standard was added to the samples and could be amplified after RT with the same primers used to detect the desired target DNA segment. However, the length of the segment amplified from the standard was different from target DNA. Sequences contained in the sample DNAs and in the internal standard were thus concomitantly amplified with the same set of primers. Quantitation could be accomplished by extrapolation of signals on a standard curve. Since both target DNA and standard DNA are amplified concomitantly in the same reaction, the difference in primer efficiency is minimized.

Another way of controlling for PCR variability has been suggested by Gilliland.[144] A competitive template which uses the same primers as those for the target DNA is co-amplified in the same reaction tube

with the sample. The amplified band from the control can be differentiated with the target since it contains a point mutation generating a restriction site absent in the normal template. With a series of dilution of this standard in sample material, the intensities of signals from each bands after digestion are compared.

Evaluation of specific HIV-1 mRNA

Selection of primers to flank the common splice donor site and acceptor sites of different genes allowed for the detection of unspliced and spliced mRNA for *tat-rev* and *nef* mRNAs[145] which could not be resolved by standard RNA analysis. One RNA segment can also be amplified with one set of primers and specific probes can identify the species of mRNA present.[148] RT-PCR can provide amplified cDNA for analysis of mRNA splice junctions or specific mRNAs.[149]

CONCLUSION

PCR is a very powerful tool for detection of viruses. Inclusion of a step with reverse transcriptase allows for the detection of HIV-1 RNA and careful selection of primers even permits discrimination between different mRNAs transcribed from the provirus. Although very useful in research, this methodology is not likely to be applied soon in every diagnostic laboratories. This especially because of the likelihood of contamination in this setting. Reporting false-positive results to clinicians and patients can have devastating consequences. Further developments in PCR will need to address this issue to make this technique less vulnerable to contamination problems.

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